The effect of melatonin administration on the oxidative status in children with autism

Mohammed BM. Al-Juboori* and Yasir Sj. Alrubaye

Department of therapeutic and clinical pharmacy, Baghdad College of Medical Sciences, Baghdad-Iraq

ABSTRACT

To determine the changes in Malondialdehyde (MDA) levels in addition to measuring the changes in the activity of superoxide dismutase (SOD) and catalase (CAT) which considered as antioxidant enzymes in autistic children receiving melatonin supplementation to evaluate its antioxidant role in autism. A follow-up study was performed in therapeutics and clinical pharmacy Department, Baghdad College of Medical Sciences, Baghdad - Iraq. The study was performed on 55 autistic children who had recruited from several private institutions specialized in autistic children care, Baghdad, Iraq between June 2018 and November 2018. The levels of melatonin, MDA, SOD and CAT were measured in the serum of 55 patients before and after receiving melatonin supplementation for three months. The results revealed statistically significant differences in the levels of melatonin, MDA, SOD and CAT between patients before and after receiving melatonin supplement. Furthermore, melatonin levels showed significant positive correlations with both SOD and CAT in addition to a significant positive correlation between SOD and CAT while MDA levels showed significant negative correlations with melatonin, SOD and CAT in autistic patients before and after receiving the supplement. Melatonin levels, CAT and SOD activity, showed to be improved significantly by melatonin supplementation with a concomitant reduction in the levels of MDA as an indicator of a decrease in oxidative stress in autistic children.

INTRODUCTION

Autism is a neurodevelopmental disorder with manifestations appear clearly within the first 36 months of the child’s life that is also related closely to many neural growth changes and that occur either pre- or postnatally (Abdulamir et al., 2018). Patients suffering from autism especially children showed a wide variety of symptoms ranged from repetitive patterns of behaviour to the more serious manifestation that includes the impairment of children’s ability to interact normally with the surrounding people as a consequence of language abnormalities besides the poor eye contact that leads to failure in social communication (Abdulamir et al., 2016b).

Furthermore, children with autism also showed an avoidance behaviour that might be caused by abnormalities in the sensory input threshold (Rose'Meyer, 2013). Autism incidence showed a sex-related pattern in which that male formed the majority of autistic patients with a ratio of 4:1 against female autistic patients (Halliday et al., 2015). The difficulties in early diagnosis of autism arise from the fact that the appearance of obvious clinical signs starts after
the age of three years, whereas previous researches the age of revealed that the signs actually appear earlier and maybe manifested at the age of 6–12 months (Rogers, 2009).

Many effectors, including neurohormones, neurotransmitters in addition to hormones and immunological mediators may participate effectively in the manifestation of clinical signs beside their crucial role in the severity and pathogenesis of autism. Melatonin considered as one of the neurohormones that play an essential role in the regulation and maintenance of circadian sleep-wake rhythm, which is synthesized mainly in the pineal gland from tryptophan (Abdulamir et al., 2016a) (Poloni et al., 2011).

Moreover, many types of research postulated that melatonin possesses several physiological functions in addition to its versatile effect on various aspects of the immune system since it serves as an immunomodulatory neurohormones. Melatonin role in controlling immune system activity still not fully understood (A et al., 2013), since that some researches stated that it has an immune-stimulant effect other studies postulated that it has anti-inflammatory activity. A review published by Carrillo-Vico and his co-workers in 2013 prove the concept of the immune-buffer activity of melatonin that behaves as either stimulant or anti-inflammatory mediator in accordance with the immune conditions (Abdulamir et al., 2016a; A et al., 2013).

Recent researches conducted on autistic children obviate that melatonin levels were abnormally decreased as a consequence of an assumed reduction in melatonin secretion. Recently, Melatonin acquires a great interest in autistic children because of its possible involvement in neurodevelopment in addition to its role in controlling sleep-wake rhythm in these patients (Tordjman et al., 2013).

Oxidative stress can be defined as an imbalance between the metabolic reactions producing free radical and antioxidant defences (enzymatic and non-enzymatic) which responsible for the protection against free radicals (Baikoussis et al., 2015). This imbalance may be caused by either free radicals overproduction such as superoxide (O₂⁻) and resulting hydrogen peroxide (H₂O₂) or a diminished in antioxidant capacity. An increase in oxygen supply (hyperoxia) may cause an elevation in free radicals leading to damage, which is considered as side effects that are manifested in a series of functional changes of many biological reactions. Highly reactive nature of free radicals cause oxidation and alteration in the normal function of many molecules such as ion pumps, receptors and enzymes and react with nucleic acid causing gene mutations that in turn cause an alteration in proteins structure and production leading to cancer (Phaniendra et al., 2015; Patlević et al., 2016).

Endogenous protective mechanism against free radicals include some antioxidant enzymes such as Superoxide Dismutase (SOD) and Catalase (CAT) that account for the first-line defence against the injurious effects of O₂ and resulting H₂O₂ by catalyzing the conversion of O₂ to H₂O₂ and oxygen (Wang et al., 2018). The reaction also can proceed spontaneously, but SOD can increase the rate of intracelular dismutation by thousand times (Sheng et al., 2014). H₂O₂ produced by SOD are reduced by glutathione peroxidase (GPx) to water or destroyed d by CAT to free oxygen and water (Wang et al., 2018).

The formation of free radical-mediated tissue damage can compromise several cell elements, causing changes in proteins or nucleic acid damage in a reaction called lipid peroxidation that changes the lipid leading to the formation of malondialdehyde (MDA) that considered as one of the most popular products of this process. So, MDA levels considered as an indicator for the extent of lipid peroxidation and also a marker for oxidative stress (Nita and Grzybowski, 2016; Ayala et al., 2014).

Several previous studies were considered melatonin and its metabolites as an antioxidant since they have several effects including ROS scavenging effect and radical-associated reactants, stimulation of SOD, GPx and CAT expression in addition to its assumed role in reducing the expression of prooxidants (Karaaslan and Suzen, 2015).

The purpose of this study was to determine the changes in the level of MDA as an indicator of oxidative damage and antioxidant enzymes such as SOD and CAT which involved in the defence mechanism against free radicals produced in autistic children receiving melatonin supplementation to evaluate its antioxidant role in autism.

**MATERIALS AND METHODS**

An experimental study was performed on 55 male autistic children recruited from several private institutions specialized in autistic children care, Baghdad, Iraq between June 2018 and November 2018. Participants’ age was ranged from 3 to 12 years (mean± SD 6.73 ± 2.71 years) and received 1 mg melatonin as daily supplementation for three months.

The children subjected to this study met the autis-
tic diagnostic criteria in accordance with the Diagnostic and statistical manual of mental disorders (5th ed.) (Association, 2013). All subjected children showed normal urine analysis results with the exclusion criteria, which include:

1. Any associated neurological disorders (such as cerebral palsy, tuberous sclerosis) and metabolic disorders (e.g. Phenylketonuria) that can alter serotonin and melatonin level of in autistic children

2. All children subjected to this study were did not receive any other medications.

The local Scientific and Review Board of the Baghdad College of Medical Sciences, Baghdad, Iraq, was accredited this study. Furthermore, parents of all subjected patients in this study were signed informed written consent of participation according to Helsinki principles.

Sample collection
A 5 ml blood samples were obtained from overnight fasting patients in plain tubes at morning (9 am). Serum separated and kept at –20°C until the time of analysis of serum melatonin, MDA CAT and SOD.

Biochemical assays
Quantitative measurement of Melatonin
ELISA Kit for the estimation of serum melatonin was obtained from Cusabio, China that employed a technique that based on a sandwich enzyme immunoassay. The microplate of the ELISA Assay kit was pre-coated with Melatonin-specific antibodies. All samples in addition to standards were applied carefully in the microplate wells, and any present melatonin was captured by the stationary antibody. Unbound substances were removed, and a biotin-linked antibody specific for melatonin then added to the microplate wells. The wells washed, and the washing step followed by the addition of avidin conjugated Horseradish Peroxidase (HRP) then washed to eliminate any unbound avidin-enzyme reagent. After that, the colour was developed by the addition of a substrate solution to the wells with an intensity proportion to the amount of melatonin that bound in the first step. Stopping solution then added to fix the intensity of the colour that developed. Colour intensity was measured spectrophotometrically at a wavelength of 450 nm. A standard curve was plotted, and the samples reading then determined by comparing them with the curve obtained.

The quantitative measurement of Malondialdehyde levels (MDA).

This measurement performed by Competitive-ELISA technique using a kit provided by AMSBIO, UK. The microplate of the ELISA Assay kit was pre-coated with Malondialdehyde. A competition occurred during the reaction between MDA in either sample or standard with a pre-coated Malondialdehyde on the microtiter plate for binding to the Biotinylated Detection Antibody specific to Malondialdehyde. Conjugated and unbound sample or standard were washed away from the microplate to remove the excess, this step followed by the addition of HRP-Streptavidin (SABC) to each well in the provided plate and incubated. A solution of TMB substrate was added to each microplate well. A sulfuric acid solution was added to terminate the enzyme-substrate reaction, and the colour obtained was measured spectrophotometrically at 450 nm. Malondialdehyde concentration in samples was quantitatively measured by platting a standard curve with which the absorbance of the samples was compared. Malondialdehyde concentration in each serum sample was expressed in ng/ml for comparison among all studied groups.

Catalase (CAT) Activity Assay
The kit used for the assay of catalase activity was provided by CELL BIOLOGUES, the USA in which the assay involved two reactions as illustrated in Figure 1. Firstly, catalase induced the hydrogen peroxide decomposition to produce water with oxygen with a rate that directly proportional to catalase concentration (See Reaction 1). A sample containing catalase was incubated in a known amount of H2O2 and allowed the reaction to proceed for one minute; during this short period, the catalase enzyme was quenched with sodium azide.

\[
\text{Reaction 1: } 2 \text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2 \text{H}_2\text{O} + \text{O}_2
\]

Figure 1: The reactions of catalase activity Kit

\[
\text{Reaction 2: } 2 \text{H}_2\text{O}_2 \text{(left over)} + \text{DHBB} + \text{AAP} \xrightarrow{\text{HRP}} \text{Quinonine Dye}
\]

Figure 2: The reactions of the assay of Superoxide Dismutase (SOD) Activity
The residual H₂O₂ that remain in the reaction mixture participate in the coupling reaction between DHBs and AAP in conjunction with an HRP catalyst (See Reaction 2). The resultant product quinoneimine dye was measured spectrophotometrically at a wavelength of 520 nm; the intensity of absorption obtained represent the concentration of this dye, which in turn correlates to the concentration of H₂O₂ remaining.

**Assay of Superoxide Dismutase (SOD) Activity**

The sensitive SOD assay kit provided by Mybiosource, USA utilizes WST-1 which reduced by superoxide anion to form a water-soluble formazan with a rate that proportionate with the activity of xanthine oxidase (XO) and reduced by SOD (as illustrated in the reaction below in Figure 2). So, the ability of SOD to inhibit this reaction can be measured spectrophotometrically.

**Statistical analysis**

Results obtained in this study were expressed firstly in a mean ± standard deviation (SD) manner and subjected to statistical comparisons by independent t-test and Pearson correlation test that assess the possible correlation among all studied parameters (Norman, 2010). The results obtained considered as significant values when **P<0.01, *P<0.05.** The statistical analysis for the results of the current study was performed by using Statistical Package for the Social Sciences (SPSS) software 20.

**RESULTS AND DISCUSSION**

The melatonin and MDA levels in addition to CAT and SOD activities were measured in patients with varying levels of autism severity before and after receiving a melatonin supplementation for three months and the results obtained in Table 1 revealed a significant increase (p<0.05) in the level of serum melatonin with a concomitant increase in the activity of CAT and SOD enzymes. On the other hand, the level of MDA showed a significant reduction after three months of melatonin supplementation.

The results illustrated in Table 2 revealed that there were significant correlations among the studied parameters that demonstrated from the significant positive correlations between melatonin and both CAT and SOD activities in addition to the significant positive correlation between SOD and CAT activities in autistic children before and after receiving the melatonin supplementation. On the other hand, the MDA level showed a significant negative correlation with the level of serum melatonin in addition to those with the activities of SOD and CAT enzymes in both pre- and post-supplementation period.

Many previous studies revealed that autistic children showed a high oxidative stress level as a consequence of low defence mechanisms against free radicals, which also reported that they participate in the pathogenesis of autism (Hernández et al., 2017; Altun et al., 2018). In the current study, the goal was to examine the possible effect of melatonin administration that used widely to correct sleep problems in affected patients on the oxidative stress through its assumed anti-oxidant activity (Karaaslan and Suzen, 2015; Cortesi et al., 2010; Sánchez-Barceló et al., 2011).

The results obtained in this study confirm the concept of anti-oxidant role of melatonin in the children suffer from autism that revealed by the significant elevation in the serum melatonin level after an administration of daily 1 mg supplement for three months that accompanied by elevation in the anti-oxidant defense mechanism enzymes activities that have a crucial impact on the oxidative status that represented by the reduction in the Malondialdehyde (MDA) level. Several explanations were reported previously for the role of melatonin in reducing oxidative stress by either direct or indirect manner that contributes to the scavenging activity of melatonin and its effect on other anti-oxidant endogenous enzymes such as CAT and SOD (Karaaslan and Suzen, 2015).

In children subjected to this study, there was an elevation in the level of MDA with a concomitant decrease in the levels of CAT and SOD enzymes before receiving the supplement that may explain the high oxidative stress which is also accompanied by low melatonin level which is in agreement with many previous types of research (Abdulamir et al., 2016a) (Tordjman et al., 2013) (Ruggeri et al., 2014). So, the possible explanation is that the defect in melatonin synthesis in autistic children may participate in reducing the activity of CAT and SOD that lead to increase the MDA levels.

After melatonin administration, Melatonin level in serum become improved significantly which is incompatible with many studies that illustrate the enhancement of sleep pattern in autistic children as an indicator of improved melatonin level in the sera of autistic patients after receiving the supplementation as reviewed by Sanchez-Barcelo and his colleagues (Sánchez-Barceló et al., 2011). The significant increase in melatonin level thought to have a role in CAT and SOD enzymes expression as stated by various studies which may contribute to its indirect anti-oxidant activity besides its direct scavenging anti-oxidant effect that lead to reduce MDA levels (Rodriguez et al., 2004; Reiter et al., 2000; Tan,
Table 1: Levels of melatonin MDA, CAT and SOD in autistic children before and after receiving melatonin supplement.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>24.23±7.19</td>
<td>28.24±6.51</td>
<td>0.002**</td>
</tr>
<tr>
<td>MDA</td>
<td>5.46±2.67</td>
<td>3.08±1.46</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>CAT</td>
<td>60.31±9.28</td>
<td>69.52±9.76</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>SOD</td>
<td>144.23±28.96</td>
<td>159.38±27.79</td>
<td>0.004**</td>
</tr>
</tbody>
</table>

**P<0.01, *P<0.05

Table 2: The Correlations among all studied groups in autistic children before and after receiving melatonin supplement.

<table>
<thead>
<tr>
<th>Before Treatment</th>
<th>After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>CAT</td>
</tr>
<tr>
<td>Melatonin r</td>
<td>-0.353**</td>
</tr>
<tr>
<td>p</td>
<td>0.006</td>
</tr>
<tr>
<td>MDA r</td>
<td>-0.314*</td>
</tr>
<tr>
<td>p</td>
<td>0.015</td>
</tr>
<tr>
<td>CAT r</td>
<td>0.443**</td>
</tr>
<tr>
<td>p</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**P<0.01, *P<0.05

1993; Galano et al., 2013a).

Furthermore, the significant correlation obtained between melatonin level and oxidative stress indicators before and after receiving the supplementation prove the possible contribution of melatonin in the prevention of oxidative damage that may participate in the pathogenesis of autism in addition to its neuro-modulatory effect that originates from melatonin capability to cross the blood-brain barrier (Abdulamir et al., 2016a).

Moreover, the results obtained elucidate that there was a significant positive correlation among melatonin, CAT and SOD in autistic patients before and after receiving melatonin while significant negative correlations were obtained between MDA on one hand and melatonin level, CAT and SOD activity on the other hand. These findings may indicate that the gradual increment in the serum melatonin may lead to increase the expression of defence mechanism enzymes that result in a reduction in the MDA level as a marker for oxidative status which is consistent with many previous reviews (Karaaslan and Suzen, 2015; Rodriguez et al., 2004).

Additionally, melatonin thought to have a protective role against free radical formation by reducing the expression of pro-oxidants, and its metabolites N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) and N(1)-acetyl-5-methoxykynuramine (AMK) have free radical scavenging ability. So, melatonin and its metabolites can serve as an efficient team of scavengers that deactivate a wide range of reactive oxygen species, under different conditions. So, the results of the current study support the previous findings that melatonin exerts continuous anti-oxidant protection in a cascade manner to scavenge reactive oxygen species (Buyukavci et al., 2006; Galano et al., 2013b).

CONCLUSIONS

In conclusion, the levels of melatonin and MDA in addition to CAT and SOD activity showed to be affected significantly by melatonin supplementation that leads finally to reduce the reactive oxygen species effect among autistic children. This study also revealed that there were significant correlations between studied parameters that can be used in future work as a powerful tool for emerging a new treatment regime that reduce the pathogenesis of autism and improve patients’ mental health in addition to reduce the sleep disorder and other miscellaneous manifestations.

ACKNOWLEDGEMENT

The authors are very gratitude to the Department of therapeutic and clinical pharmacy at Baghdad College of Medical Sciences for providing the facilities for the achievement of this work. We are very grateful to Al-Dhuha, Al-Safa and Al-Rahman centres for Auspices of Autistic and Lumpen children for their
collaboration in the success of this work

**Authors Contributions**

Mohammed BM. Al-Juboori was responsible for the collection of samples from patients before and after the administration. Yasir SJ. Alrubaye was responsible for the biochemical work in addition to his role in the follow-up of patients during the period of drug administration.

**REFERENCES**


Rogers, S. J. 2009. What are infant siblings teaching us about autism in infancy? Autism Research,


